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Dual Role of Hypoxanthine in the Reoxygenation of Hypoxic Isolated Rat Hearts

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M. Tarantola, R. Motterlini, M. Beretta and M. Samaja. Dual Role of Hypoxanthine in the Reoxygenation of Hypoxic Isolated Rat Hearts. *Journal of Molecular and Cellular Cardiology* (1991) **23**, 77–82. In the reoxygenated hypoxic heart, hypoxanthine is either oxidized by xanthine oxidase with production of toxic oxygen species or salvaged for the ATP pool by hypoxanthine-guanine phosphoribosyl transferase. To characterize the repartition of hypoxanthine between the two pathways, we have subjected rat hearts to 20 min hypoxia and monitored the recovery (ventricular, end-diastolic and coronary pressures, and the contraction rate) during the reoxygenation (30 min) in the presence of either hypoxanthine or guanine alone, or both. The rate–pressure product recovered 78% of the pre-hypoxia values in hearts reoxygenated with 100 μM hypoxanthine and 80% in hearts recoxygenated with 100 μM guanine, in contrast to 49% in the presence of both hypoxanthine and guanine (100 μM each). Thus, it is likely that hypoxanthine is salvaged when present alone and is oxidized generating the reperfusion injury when the salvage is prevented by guanine that competes with hypoxanthine from the same site of hypoxanthine-guanine phosphoribosyl transferase. The functional impairment was slower when hypoxanthine was replaced by xanthine, and was eliminated by superoxide dismutase and catalase, indicating that the injury is caused by toxic oxygen species generated from hypoxanthine and xanthine oxidase. These data suggest that the salvage pathway may be critical in preventing the reperfusion injury in hypoxic hearts.

Key Words: Reperfusion injury; Reoxygenation injury; Oxygen-derived free radicals; ATP catabolism; Hypoxanthine; Xanthine oxidase; Purine salvage; Heart recovery.

Introduction

The role of hypoxanthine (HX)[1] and xanthine oxidase (XO) in the pathogenesis of the injury in hearts subjected to ischemia/reperfusion is known (McCord, 1985). Briefly, the O_2 shortage leads to cell energy depletion, increases intracellular HX (Jennings and Steenbergen, 1985) and induces the conversion of xanthine dehydrogenase into XO (Parks and Granger, 1986). At the reperfusion, HX reacts with O_2 and XO releasing O_2-derived free radicals and triggering a series of events referred to as the reperfusion injury (Chambers *et al.*, 1985; Downey *et al.*, 1988; Granger *et al.*, 1986). However, there is evidence for an alternative metabolic pathway by which HX is salvaged for the adenine nucleotide pool by HX-guanine phosphoribosyl transferase (HGPRT) improving the recovery of the heart from ischemia (Maguire *et al.*, 1972; Manfredi and Holmes, 1985; Lasley *et al.*, 1988), but little is presently known on the repartition of HX between the oxidation and the salvage. The metabolic scheme under study is sketched in Figure 1.

To study these apparently contradictory mechanisms and to test the scheme of Figure 1, we have subjected isolated rat hearts to hypoxia and monitored their recovery in the presence of one or more of the diffusible purines, i.e., HX, guanine (GUA) or xanthine (XAN), in the perfusion buffer. The purines were thus removed from or loaded into the myocardium along their concentration gra-

[1] List of abbreviations: CP, coronary pressure; EDP, end-diastolic pressure; GUA, guanine; HGPRT, hypoxanthine-guanine phosphoribosyl transferase; HR, heart rate; HX, hypoxanthine; LVDP, left ventricle developed pressure; XAN, xanthine; XO, xanthine oxidase.

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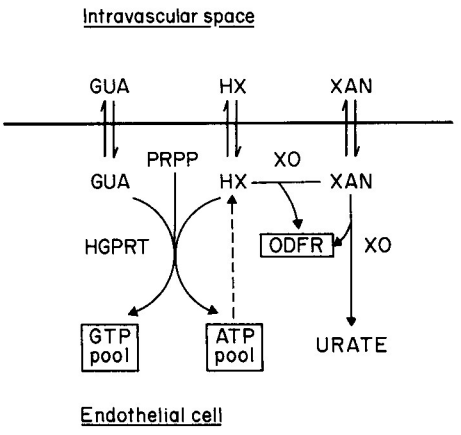

FIGURE 1. Scheme of the metabolic pathways involving hypoxanthine in the myocardium endothelial cell exposed to hypoxia and reoxygenation. Abbreviations: GUA, guanine; HGPRT, hypoxanthine-guanine phosphoribosyl transferase; HX, hypoxanthine; ODFR, oxygen-derived free radical; PRPP, 5-Phosphoribosyl-1-pyrophosphate; XAN, xanthine; XO, xanthine oxidase.

dient and their intracellular concentration was constant during the heart recovery. XAN and GUA helped to characterize the metabolism of HX because XO catalyzes the oxidation of XAN with half yield of free radicals, and GUA competes with HX for the same site of HGPRT increasing the availability of HX for the XO reaction. We provide data suggesting that HX is preferably salvaged in the post-hypoxic heart, being oxidized only when the salvage pathway is limited.

Materials and Methods

Chemicals

Stock of 0.1 M solutions of HX, XAN and GUA (Sigma Chemicals, St. Louis, MO, USA) were prepared dissolving the purines in 0.1 M NaOH at room temperature (no pH changes were detected when perfusing hearts with such solutions). Superoxide dismutase and catalase were obtained from Boehringer Biochemia Robin (Milano, Italy) and used without any further treatment. All other reagents were purchased from Farmitalia Carlo Erba (Milano, Italy) and were analytical grade.

Heart perfusion

Ad libitum fed male Sprague–Dawley rats weighing 250–270 g were anesthesized by i.p. injection of heparinized 20% (w/v) urethan (1 ml/100 g body weight). The animals were thoracotomized, the heart was excised and placed in ice-cold isotonic saline (ischemia < 1 min), and then the aorta was mounted onto a stainless-steel 1.8 mm o.d. cannula. The heart was perfused accordingly to the Langendorff's technique with 115.6 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 0.5 mM EDTA, 1.2 mM Na_2SO_4, 28.5 mM $NaCHO_3$, 3 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 16.6 mM glucose. The buffer was equilibrated with either O_2/CO_2 or N_2/CO_2 (94%/6% for both). The pH was 7.4 at 37°C. A roller pump (Watson Marlow mod.503-S, Falmouth, England) delivered the buffer at 15 ml/min through a 8 μm pore size, 47 mm diameter polycarbonate filter (Nuclepore Corp., Pleasanton, CA, USA), a heat exchanger, and the aortic cannula. An infusion pump (Razel Scientific Instrument mod.A–99, Stanford, CT, USA) delivered HX, GUA, and XAN at 15 μl/min. An additional Razel pump delivered superoxide dismutase and catalase at 0.1 ml/min. The flow of the infusion and of the roller pumps was the same throughout and was routinely checked by weighting the perfusate at fixed time intervals, thus the final concentration of the purines in the perfusate was 100 μM each, while that of superoxide dismutase and catalase was 40 IU/ml and 104 IU/ml, respectively. The coronary perfusion pressure was monitored by a pressure transducer (Harvard Apparatus mod.52–9966, Natick, MA, USA) placed above the aortic cannula. A saline-filled latex balloon was inserted into the left ventricle through the mitral valve and connected to an additional Harvard transducer to monitor the mechanical functions. Finally, a teflon cannula was inserted in the pulmonary artery to collect the perfusate.

Measurements

The signal from the transducer coupled to the intraventricular balloon was analyzed for the waveform either by a digital oscilloscope (Nicolet Scientific Instruments mod.3091, Madison, WI, USA) or by a signal conditioner that outputs the end-diastolic pressure (EDP) the left ventricular developed pressure (LVDP), and the contraction rate ($+dP/dt_{max}$). These signals and the coronary

perfusion pressure (CP) were monitored on a strip chart recorder (Kipp & Zonen BD 101, Delft, Holland). An integrated index of the cardiac performance was computed as the rate-pressure product (LVDP × heart rate).

Experimental protocol

The spontaneously contracting hearts were allowed to stabilize with the oxygenated buffer for 30 min. The EDP was set to 7–8 torr and the baseline values were recorded. The hearts that were unable to perform at a predefined metabolic level (LVDP <80 torr, or PO_2 of the perfusate <140 torr) were not admitted to the study protocols. To induce hypoxia, the buffer was switched to the O_2-free one. After 20 min, the hearts were perfused with the oxygenated buffer in the presence of various substrates, and the recovery was monitored for 30 min. The hearts were divided into five groups depending on the substrate present during the reoxygenation: GUA, 100 μM GUA ($n = 6$); HX, 100 μM HX ($n = 6$); GUA + HX, 100 μM GUA + 100 μM HX ($n = 6$); GUA + XAN, 100 μM GUA + 100 μM XAN ($n = 5$); SOD/CAT, 100 μM GUA + 100 μM HX + 40 IU/ml superoxide dismutase + 104 IU/ml catalase ($n = 5$).

Statistics

For statistical evaluation, analysis of variance was used (1-way classification). When two means were compared, Student's unpaired *t*-test was used and $P = 0.05$ was considered not significant (NS). The Wilk-Shapiro test of normality was used previously to the Student's *t*-test to decide whether the parametric test could be run. All data are expressed as mean ± S.E.

TABLE 1. Values of the relevant parameters in the Langdendorff-perfused rat hearts ($n = 28$) before the onset of hypoxia

Parameter	Mean	S.E.
Coronary pressure, torr	92.0	4·3
End-diastolic pressure, torr	7.2	0.3
Left ventricular developed pressure, torr	117.3	3.0
dP/dt_{max}, torr/s	4745	160
Heart rate, min^{-1}	289	15
Rate-pressure index, Ktorr/min	33.8	5.5

Results

The changes of EDP, LVDP, and CP were less than +1, −5, and +5 torr, respectively, over a 90-min perfusion with the oxygenated buffer. The values of the relevant parameters before the onset of hypoxia are in Table 1. As expected, the heart function was severely depressed in hypoxia and no spontaneous recovery was possible in hearts with LVDP <20 torr or EDP >70 torr.

Table 2 reports the change of the heart function 30 min after the reoxygenation with respect to the pre-hypoxia values. The analysis of variance, performed for each parameter,

TABLE 2. Mean and S.E. of the changes of the parameters with respect to the pre-hypoxia values in hearts made hypoxic for 20 min and reoxygenated for 30 min in the presence of various substrates (100 μM each)

	GUA	HX	GUA + HX	GUA + XAN	SOD/CAT
Coronary pressure, torr	31.0 ± 4.3	7.7 ± 4.2	95.0 ± 31.3	89.0 ± 17.2	−13.0 ± 4.2
End-diastolic pressure, torr	11.4 ± 7.0	5.8 ± 2.3	66.6 ± 11.8	53.8 ± 10.7	−0.6 ± 0.4
Left ventricular pressure, torr	−26.0 ± 5.9	−30.8 ± 2.5	−58.8 ± 15.8	−59.0 ± 7.7	−23.8 ± 5.0
dP/dt_{max}, torr/s	−812 ± 254	−870 ± 205	−2182 ± 597	−2262 ± 301	−534 ± 251
Heart rate, min^{-1}	6.0 ± 6.7	14.7 ± 11.0	2.2 ± 7.4	−3.0 ± 15.1	17.0 ± 8.6
Rate-pressure index, Ktorr/min	−6.8 ± 2.0	−7.3 ± 1.8	−17.2 ± 4.5	−16.4 ± 2.0	−5.7 ± 1.0

shows that the changes in the five groups under study are significant for the CP ($P < 0.001$), EDP ($P < 0.001$), LVDP ($P < 0.007$), dP/dt_{max} ($P < 0.002$), but not for the heart rate. Thus, we have considered the rate-pressure product (LVDP × HR) as an integrated index of the heart performance, and the analysis of variance shows that its changes are significant ($P < 0.02$).

The myocardial performance after the reoxygenation was lower in both the GUA and HX groups with respect to the pre-hypoxia value for all the parameters. The rate-pressure product recovered 80% and 78% in hearts reoxygenated with 100 μM GUA and 100 μM HX, respectively. The two groups are not significantly different with the exception of the CP, higher in the GUA group ($P < 0.001$). When both GUA and HX were present during the reoxygenation (GUA + HX group), the depression was more severe than in the GUA and in the HX groups for CP ($P < 0.03$), EDP ($P < 0.005$), LVDP ($P < 0.05$), dP/dt_{max} ($P < 0.04$) and LVDP × HR ($P < 0.05$). The rate-pressure index indicated that the heart function in the GUA + HX group at the end of the reoxygenation was 49% of the pre-hypoxia level.

In some experiments, HX was replaced by equimolar XAN (GUA + XAN group). The recovery at the end of the reoxygenation of LVDP × HR in this group was similar to that of the GUA + HX group (51%) and there are no significant differences between the GUA + HX and the GUA + XAN groups in the various parameters. However, the time course of the recovery of LVDP, $+dP/dt_{max}$ and EDP in the GUA + XAN group is between those of the GUA + HX and the GUA groups (Fig. 2).

In other experiments, the hearts were reoxygenated with GUA, HX and in the presence of 40 IU/ml superoxide dismutase and 104 IU/ml catalase (SOD/CAT group). The recovery of LVDP × HR was 85% and not significantly different from the GUA and HX groups for LVDP, dP/dt_{max} and LVDP × HR. However, the CP and EDP were lower in the SOD/CAT group than in the GUA and HX groups ($P < 0.02$ for both).

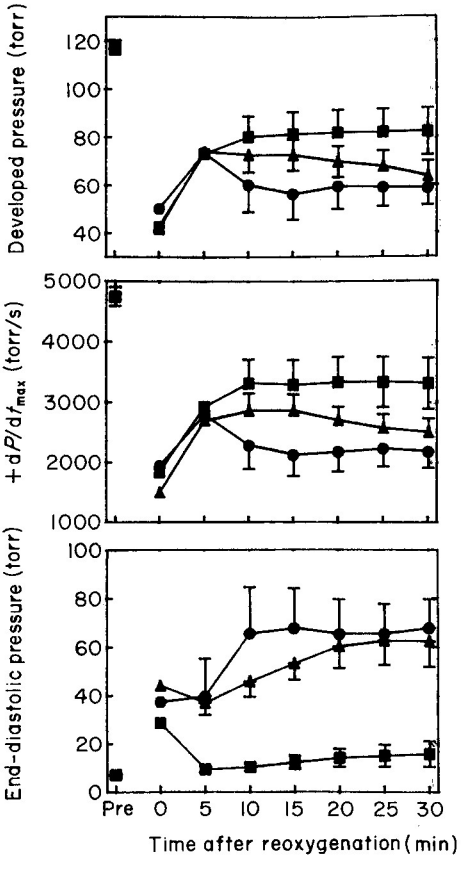

FIGURE 2. Kinetics of the recovery from 20 min hypoxia of left ventricular developed pressure, $+dP/dt_{max}$, and end-diastolic pressure in the presence of GUA (■), GUA + XAN (▲), and GUA + HX (●). The bars represent the S.E.

Discussion

The main finding of this study is that the recovery of the isolated rat heart from hypoxia was essentially the same in hearts reoxygenated with either HX or GUA, and was significantly impaired in the presence of both HX and GUA. The time course of the impairment was slower when XAN replaced HX, and the recovery improved in the presence of superoxide dismutase and catalase.

With continuous perfusion of the hearts with either the O_2-free or the oxygenated buffers, diffusible purines could permeate across the endothelial cell membrane along their concentration gradient. Indeed, HX is uptaken by the mild-ischemic rat myocardium with virtually no concentration gradient (Harmsen *et al.*, 1984) and although we are not aware of data on the permeability of XAN and GUA, there is no valid reason to believe that it is not so for these purines as well since

closely related molecules as inosine, adenosine and allopurinol cross freely the membrane (Harmsen *et al.*, 1984; Dow *et al.*, 1987, Lasley *et al.*, 1988). Therefore, it was assumed that the myocardium was loaded with purines at the same concentration of the perfusing buffer, and that purines reacted with the relevant enzymes, i.e., cytoplasmic XO, major source of oxygen radicals in the reperfusion injury (Chambers *et al.*, 1985; Downey *et al.*, 1988; Granger *et al.*, 1986), and HGPRT, that uptakes both GUA and HX for the salvage to the GTP and ATP pools. Table 3 summarizes the most relevant properties of these enzymes.

On reoxygenation, HX may undergo two metabolic pathways: (1) oxidation by XO with production of oxygen radicals and impairment of the heart function; and (2) salvage to the ATP or GTP pools by HGPRT. The ribosylation of HX by purine nucleoside phosphorylase is not important in the myocardium (Manfredi and Holmes, 1985). Since the activity of HGPRT is higher than that of XO (Table 3), HX is more likely salvaged than oxidized if the salvage is not prevented, and the similar myocardial function in the GUA and HX groups indicates that there are no substantial differences between these two groups for the irreversible injury. However, in the presence of both GUA and HX, a different metabolic pattern occurs: (1) GUA inhibits the salvage of HX competing for the same site of HGPRT with a slightly higher affinity (Table 3); (2) The GTP pool is restored faster with lower need for the cell to uptake HX for the same purpose; (3) The cell stores of 5-phosphoribosyl-1-pyrophosphate, perhaps the main regulator of the salvage (Dow *et al.*, 1987), are depleted faster. The overall consequence of these events is the larger availability of HX for the oxidative pathway as reflected by the greater impairment in the hearts of the GUA + HX group. It seems therefore that HX is involved in the reoxygenation injury only when the salvage pathway is inhibited by GUA.

The critical role of the repartition of HX between the two pathways in the reperfusion injury was addressed replacing HX with XAN, that is not salvaged and produces half oxygen radicals than HX (GUA + XAN group). If the myocardial injury is linked to the XO reaction, its severity is expected to be lower in this group. Figure 2 shows that the time course of the recovery in the GUA + XAN group is slower than in the GUA + HX group. The significativity of the differences between the two groups at the end of the reoxygenation is poor because of the high concentration of purines with respect to the available XO and the K_M values.

The role of the oxygen radicals was assessed applying the same protocol of the GUA + HX group in the presence of superoxide dismutase and catalase, that lower the activity of certain oxygen radicals (SOD/CAT group). The impairment, correspondingly lower than in the GUA + HX and GUA + XAN groups and similar to the GUA and HX groups, confirms the involvement of oxygen-derived free radicals as well as the observation that the impairment in the GUA + HX group is similar to that driven by the oxygen radicals generated by electrolysis in rabbit hearts (Stewart *et al.*, 1988): CP and EDP increase, and the vascular permeability,

TABLE 3. Reactions catalyzed and some properties of the enzymes xanthine oxidase (XO) and hypoxanthine-guanine phosphoribosyl transferase (HGPRT).

	XO	HGRPT
Reactions	$HX + O_2 \rightarrow XAN + ODFR$	$HX + PRPP \rightarrow IMP + P_i$
	$XAN + O_2 \rightarrow urate + ODFR$	$GUA + PRPP \rightarrow GMP + P_i$
K_M (HX)[a]	10	2.4–11
Activity	0.012–0.073[b]	8.7[a]
K_M (GUA)[a]	—	1.8–4

The K_M values are expressed in μM, and the activities in mU/mg wet wt (Maguire *et al.*, 1972[a], and Park and Granger, 1986[b]). Other abbreviations: GUA, guanine; HX, hypoxanthine; PRPP, 5-phosphoribosyl-1-pyrophosphate; XAN, xanthine.

or $+dP/dt_{max}$ (Laine, 1987), decreases with little impairment of the heart rate and the oxygen uptake (not shown). It appears therefore that the reoxygenation injury led by HX is mediated by the oxygen radicals.

In conclusion, the recovery of isolated hearts from hypoxia seems partially dependent on the repartition of HX between the oxidation and the salvage. We propose that HX is normally salvaged contributing to restore the ATP pool. However, in the presence of high concentrations of GUA, competitor of HX for HGPRT, and possibly other factors that decrease the HGPRT activity such as intracellular loss of ribose and 5-phosphoribosyl-1-pyrophosphate, the salvage is impaired and larger amounts of HX are oxidized by XO triggering the ODFR-derived injury. The relevance of these phenomena in normo- and hypo-thermic ischemia/reperfusion is still to be evaluated.

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